AD_____

Award Number: W81XWH-08-1-0503

TITLE: Noninvasive Subharmonic Pressure Estimation for Monitoring Breast Cancer Response to Neoadjuvant Therapy

PRINCIPAL INVESTIGATOR: Flemming Forsberg, Ph.D.

CONTRACTING ORGANIZATION: Thomas Jefferson University
Philadelphia, Pennsylvania 19107

REPORT DATE: September 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2009	Annual (September 1, 2008 – August 31, 2009)

4. TITLE AND SUBTITLE
Noninvasive Subharmonic Pressure Estimation for Monitoring Breast Cancer Response to Neoadjuvant Therapy

5. FUNDING NUMBERS
W81XWH-08-1-0503

6. AUTHOR(S)
Flemming Forsberg, Ph.D.
Email: Flemming.Forsberg@jefferson.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Thomas Jefferson University
Philadelphia, Pennsylvania 19107

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Neoadjuvant chemotherapy is currently the standard of care for locally advanced breast cancer (LABC). Monitoring tumor response is advantageous for patients. This project aims at establishing noninvasive monitoring of neo adjuvant chemotherapy in the breast u sing subharmonic aided p ressure estimation (SHAPE) to estimate the inte rstitial fluid pressure (IFP) in LABC.

To date, in vitr o experiments with 2 ultra sound contrast agents (Definity a nd Sonazoid) have showed an inverse linear relationship between the change in subharmonic amplitude and hydr ostatic pressure (r2 = 0.76 –0.91, p < 0.01) o ver the p ressure range associated with breast tumors (0 – 47 mmHg). These results indicate that the use of SHAPE for noninvasive evaluation of the IFP in breast lesions is feasible with both Definity and Sonazoid. Moreover, software for ana lyzing RF data fro m a Sonix RP scann er to pro duce SHAPE pressure estimates has been successfully developed. However, difficulty in aligning the in vitro setup has delayed the project by approximately 6 months.

14. SUBJECT TERMS
Breast Cancer, Ultrasound Imaging, Ultrasound Contrast Agent, Pressure Estimation, Neoadjuvant chemotherapy

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

4 INTRODUCTION

In the United States, close to 5 – 20 % of newly diagnosed breast cancer and 10 – 30% of all primary breast cancer is diagnosed as locally advanced breast cancer (LABC) [1, 2]. Neoadjuvant chemotherapy (systemic preoperative chemotherapy) is currently the standard of care for LABC [3, 4]. When compared with adjuvant chemotherapy (postoperative therapy), neoadjuvant chemotherapy yields similar results for both overall survival (70% for both) and disease-free survival (53% adjuvant, 55% neoadjuvant) [5]. Thus, the postponement of surgery does not affect the outcome of the treatment [5, 6]. In addition, neoadjuvant chemotherapy offers considerable benefits to the patient as the treatment can shrink the tumor and even in some cases offer complete pathologic response [3, 7]. This reduction in tumor size increases the possibility of breast conservation [3, 5-7]. Maximizing the conservation of breast tissue can be of great personal importance for the self-esteem and quality of living of the patient [6]. Neoadjuvant chemotherapy can also offer an early indication of the patient's response to chemotherapy. Consequently, monitoring tumor response to neoadjuvant therapy gives the possibility of adjusting the treatment if the patient is responding poorly or not at all resulting in substantial advantages for the patient [3, 6]. This project aims at establishing noninvasive monitoring of neoadjuvant chemotherapy in the breast using subharmonic aided pressure estimation (SHAPE; U.S. Patent 6,302,845).

Generally interstitial fluid pressure (IFP) is 10-30 mmHg higher in cancerous tissue than in normal tissue although values of up to 60 mmHg have been recorded [8, 9]. Similarly, IFP in breast cancer tumors has been shown to be higher than that of surrounding breast tissue [9]. This increase is believed to be due to vascularity, fibrosis and difference in the interstitial matrix in tumors and it can result in poor transport of therapeutic drugs to tumors [8]. Taghian et al. used a wick-in-needle technique to monitor the IFP of breast cancer before and after neoadjuvant chemotherapy with two drugs used consecutively [10]. When used as a first drug Paclitaxel decreased the IFP by 36% ($p=0.02$) whereas with Doxorubicin as a first drug there was only 8% reduction ($p=0.41$). As this was a hypothesis-generating study they did not show any outcome related to the relationship between IFP and therapy response [10]. However, the level of IFP has been shown to predict disease free survival for cervix cancer (34% disease free survival (DFS) if IFP > 19 mmHg, 68% DFS if IFP < 19 mmHg ($p = 0.002$)) [11]. Thus, the level of interstitial fluid pressure (IFP) in breast cancer tumors could potentially be used to monitor the response to neoadjuvant chemotherapy.

Contrast agents have been used for two decades to improve visualization in ultrasound (US) imaging as they enhance the difference in reflectivity between tissues [12]. Because of the difference in compressibility between the medium and the microbubble any changes in pressure induce changes in the size of the microbubble [13]. This in turn affects the reflectivity and resonant frequency of the bubble [13, 14]. In subharmonic imaging (SHI) pulses are transmitted at a frequency f_0 and the echoes are received at half that frequency $f_0/2$. SHI has been showed to be a feasible option for contrast enhanced imaging due to subharmonic generation by contrast agents and limited subharmonic generation in tissues [15]. Our group came up with a novel technique, SHAPE, utilizing

microbubbles and the subharmonic amplitude of the scattered signal [13]. We showed that there is a linear relationship between the hydrostatic pressure and the subharmonic amplitude. We propose the use of SHAPE to monitor treatment response by noninvasively measuring the IFP in breast tumors. This offers several benefits to the patient. As opposed to the wick-in-needle technique SHAPE is noninvasive and does not inflict pain. Furthermore, it allows for an early indication of responders vs. non-responders and thereby makes adjustments to therapy easier. Moreover, SHAPE has been shown to have a favorable signal-to-noise ratio so the subharmonic amplitude is not affected by background noise [13].

The optimal contrast agent and acoustic parameters for SHAPE will be established using *in vitro* pulse-echo measurements. The SHAPE algorithm will then be designed and implemented on a commercial, state-of-the-art US scanner for *in vivo* IFP measurements. A similar algorithm has already been set up for cardiac SHAPE and thus only a few adjustments need to be made to implement SHAPE for breast tumors making this very cost-effective. The *in vivo* experiments will be twofold. First, athymic, nude, female rats will be implanted with SKBR3, MCF-7 or BT474 human breast cancer cells and SHAPE used to measure IFP and calibrated by comparing the SHAPE results to IFP measurements obtained with an invasive, intra-compartmental pressure monitor as the gold standard. After calibration, human xenograft breast tumors in athymic, nude, female rats will be used to evaluate the ability of SHAPE to track changes in IFP by studying before and after administration of a chemotherapy agent (paclitaxel).

Our group has proposed that SHAPE and contrast enhanced US imaging can be used to measure the IFP in LABC tumors, thus, making it possible to noninvasively monitor the tumor response to neoadjuvant chemotherapy. This method would be a considerable improvement from the wick-in-needle technique currently used for IFP measurements in LABC and allow for individualized treatments options.

5 BODY

The hypothesis of this project is that IFP in breast tumors can be measured noninvasively using SHAPE and contrast enhanced US thus improving the monitoring of neoadjuvant chemotherapy. To investigate this prospect, *in vitro* pulse-echo experiments will be conducted to investigate this prospect and find the optimal contrast agent for SHAPE. These results will then be used to implement SHAPE on a commercial scanner. The scanner will be used for *in vivo* studies on 201 rats with tumor xenografts in order to calibrate and evaluate SHAPE's ability to monitor response to neoadjuvant chemotherapy. The specific tasks of the project (as presented in the original Statement of Work) can be found in Appendix I.

First an outline of the methods applied will be given followed by a presentation of the results to date. Finally, the conclusions and future directions of the research will be discussed.

5.1 Methods

In vitro experiments

A pulse-echo system , si milar to the one pr eviously developed by our group [13], was constructed to test different types of contrast agents for use with low-frequency SHAPE under support by the NIH and the AHA. Henc e, it was possible to use this setup to investigate high frequency SHAPE at very limited expense to this grant. A programmable function generator (8116A; He wlett Packard, S anta Clara, CA) produced pulses, which were supplied to the transm it transducer af ter am plification in a broadband 50 dB RF power am plifier (325L A; ENI, Rocheste r NY). Received m icrobubble signals were amplified with a low noise RF am plifier (5052 PR; Panam etrics, W altham, MA), digitized and processed (with the built-in FFT function) using a digital oscilloscope (9350AM; LeCroy, Chestnut Ridge, NY). The a mplitude of the harm onic and subharmonic signal components was obtained from spectra averaged over 64 sequences. Command delivery and data transfer was cont rolled by LabView (National Instrum ents, Austin, TX). The subharmonic amplitude at different static pressures was measured using a sealed water tank capable of withsta nding pressure change s over 200 mmHg. Single element transducers with center frequencies of 4 to 12 MHz were us ed as transmitter and receiver. Th e tank was immersed in a wate r bath at roo m tem perature (25°C). The pressure inside the tank was m onitored by a pressure gauge (OME GA Engineering, Stamford, CT). An inlet and an outle t on the tank were constructed for injecting microbubbles and for applying extra hydrostatic pressure.

In order to find the optim al contrast agen t for high-frequency SHAPE changes in the amplitudes of the first, second and subharm onic were measured for two different contrast agents; Definity (Lanth eus Med ical Im aging, N Billerica, MA) and Sonazoid (GE Healthcare, Oslo, Norway) at pressures from 0 to 47 mmHg (to simulate the IFP in breast tumors). Furtherm ore, the frequency and acous tic pre ssure were vari ed from 5.7 to 9.3 MHz and 0.7-1.2 MPa respectively to determ ine the optimal acoustic param eters. After data retrieval the am plitude of the first, second and subharmonic was extracted using MATLAB 7.0.4 (Mathw orks, Natick, MA). Thre e m easurements were acquired at each setting and linear regression analysis used to dete rmine the r elationship between hydrostatic pressure and change in amplitude for the firs t, second and su bharmonic. All statistical analysis was conducted using Stat a 9.0 (Stata C orporation, College Station, TX).

Moreover, a novel, sim ulation m odel of the dynam ics of a n encapsulated m icrobubble contrast agent, developed as part of a previous DOD s upported project [16], was modified in order to account for a mbient pressure variations and different shell parameters to establish the optim al c ontrast m icrobubble for SHAPE. A nonlinear extension of the origin al visco elastic m odel was pursued by considering a quadratic elasticity model where the interf acial elasticity vary linearly with area fraction as wel l as an exponential model (i.e., the elasticity vary exponentially).

In vivo experiments

Our group has worked in partnership with Ul trasonix Medical Corporation to im plement SHAPE for cardiac use on a state-o f-the-art commercial scanner Sonix RP (Ultrasonix

Medical Corporation, R ichmond, BC, Canada) with a phased array (PA4-2). Several experiments have been carried out in canin es to investigate ca rdiac SHAPE supported by funding from the AHA. RF data f rom these experiments was analyzed off-line using Matlab. The software developed by our group for this analysis is not site-specific and will also be used to analyze the data we will acquire from *in vivo* breast SHAPE in rats as part of this project.

Finally, an opportunity to collaborate with Dr. Gregory Czarnot a of Sunnybrook Health Sciences Centre in Toronto, Canada has arisen. Dr. Czarnota is currently conducting *in vivo* research on cell death detect ion in women with LABC usin g Definity and he offered to acquire RF data from his patients for S HAPE at no cost to this project. Thus , preliminary tests using the m odified Soni x RP scanner were conducted to provide guidance on the optim al frequencies for *in vivo* hum an st udies to Dr. Czarnota. Two different probes, L14-5/38 and L9-4/38, were tested at freq uencies ranging from 3 – 10 MHz at a 0 dB power le vel both with and witho ut pulse inv ersion (PIHI). For this s et of experiments 0.38 m l/l of Definity were in jected into an open w atertank an d the ultrasound probe suspended into the tank fo r m easurements. The agent was kept in suspension with a m agnetic st irrer. Three m easurements (5 s scan s) of B- mode/RF imaging were taken for each frequency setting. The data was then transferred to a PC for offline analysis (using Matlab).

5.2 Results and Discussion

In vitro experiments

Over the pressure range of 0 – 47 mmHg (simulating th e IFP in br east tum ors) both Definity and Sonazoid showed an inverse linear re lationship betwee n the chang e in subharmonic am plitude and hydrostatic pres sure ($r^2 = 0.76–0.91$, $p < 0.01$). This is consistent with previous results reported by our group [13]. An exam ple of the subharmonic amplitude for Sonazoid at 0 mmHg and 47 mmHg can be seen in F igure1 (transmitting frequency 7.5 MHz and acoustic pressure 0.7 MPa). Th is process has been markedly more tim e-consuming then origina lly envisaged, due to difficulty in aligning the sing le elem ent tran sducers at these hi gher frequencies (com pared to our original efforts e.g., in [13]) and this issue has delayed the project by approximately 6 months.

Definity was the m ore sensitive ag ent w ith a m aximum decrease 3.86 dB over 0-47 mmHg at an im aging frequency of 6.6 MHz and acoustic pressure 1.1 MPa (see F igure 2a) whereas Sonazoid showed a m aximum decrease of 2.40 dB at 7.5 MHz and 0.7 MPa (see Figure 2b). Table1 lists the results for Definity at d ifferent acoustic pressures and transmitting frequencies. These resu lts ind icate that the use of SHAPE for noninvasive evaluation of the IFP in breast lesion s is feas ible with both Definity and Sonazoid, given in vitro co rrelation coefficients reaching 0.9 1. Initia l results were reported a t an international conf erence [17] and the m ore complete r esults will b e presented a t the RSNA in December, 2009 [18]. These efforts re present the partial fulfillment of tasks 1a, 1d and 1f in the original Stat ement of W ork (SOW). Future *in vitro* studies will include further experim ents at lower (4.0 - 5.7 MHz) and higher (9 .3 – 12.0 MHz) transm itting frequencies as well as a wider acoustic pressure range before moving on to the *in vivo* rat xenograft measurements planned as task 3 in the original SOW.

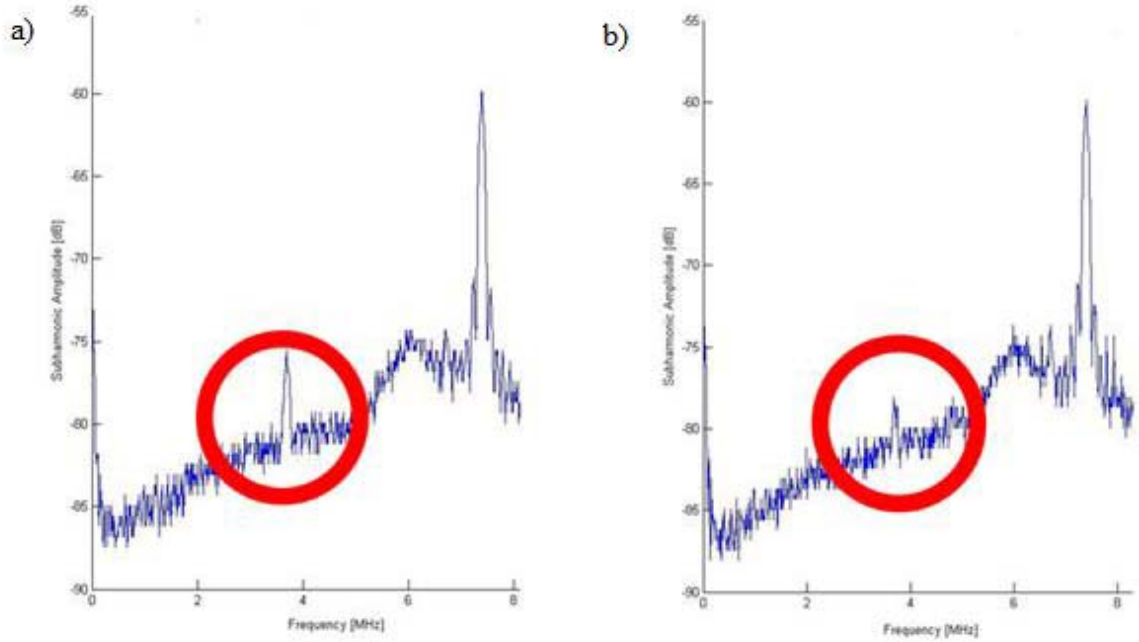

Figure 1. *Comparison of the subharmonic amplitude (circle) measured with Sonazoid at a) 0 mmHg and b) 47 mmHg pressures.*

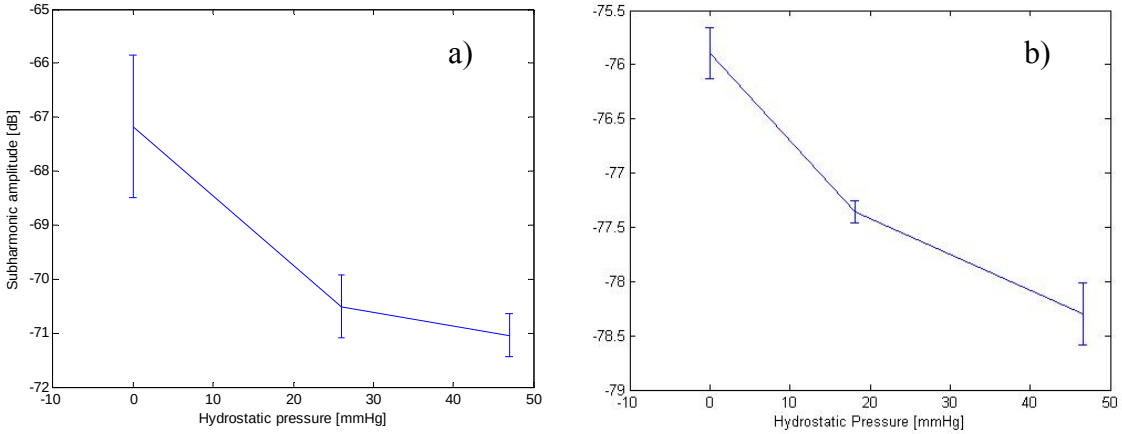

Figure 2. *Changes in subharmonic amplitude over the pressure range encountered in LABC with a) Definity showing a decrease of 3.86 dB at an imaging frequency of 6.6 MHz and acoustic pressure 1.1 MPa, and b) Sonazoid showing a decrease of 2.40 dB at an imaging frequency of 7.5 MHz and acoustic pressure 0.7 MPa.*

The previously developed sim ulation m odel of the dynam ics of an encapsulated microbubble contrast agent was modified to include nonlinear extensions of the viscoelasticity. The intent was to better acc ount for the experimentally observed changes in subharm onic signal am plitudes as a functio n of hydrostatic pres sures. However, results to date have not been able to docum ent this behavior adequately [19] and fur ther refinements are being pursued. This is the commencement of tasks 1b, 1c and 1e.

Table 1. Maximum reduction in subharmonic amplitude of Definity at different transmitting frequencies and acoustic pressures.

Transmitting frequency (MHz)	Acoustic pressure (MPa)	Subharmonic decrease (dB)
5.7	0.9	2.73
6.6	1.1	3.86
7.5	1.1	2.7
8.4	0.9	1.38
9.3	1.2	1.19

In vivo experiments

Software has been developed to analyze RF data from the Sonix RP scanner and produce cardiac SHAPE pressure estimates (although this software is equally applicable to RF data from breast tumors) [20]. So far data from 8 canines have been analyzed with excellent results to provide *in vivo* proof-of-concept of the feasibility of SHAPE. An example of the pressure measured with a pressure catheter compared to the noninvasive SHAPE results can be seen in Figure 3 for the left ventricle ($r^2 = 0.78$; $p < 0.001$) and in Figure 4 for the right atrium ($r^2 = 0.66$; $p < 0.001$), respectively.

Further developments are ongoing and currently the main focus is on establishing the best method to extract the subharmonic signal components from the frequency spectrum. Moreover, a patent application has been submitted based on this development effort. Thus, considerable time has been spent on developing this software and this effort represents the commencement of task 2b.

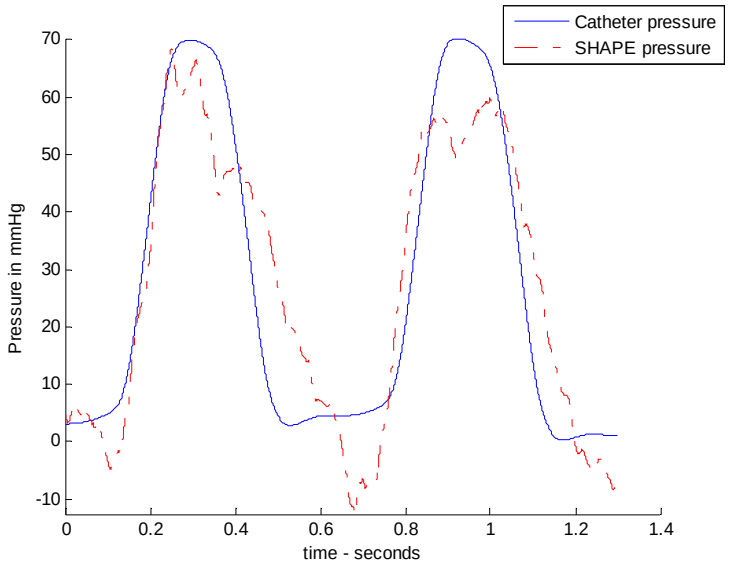

Figure 3. *Comparison of the catheter pressure (blue) and SHAPE pressure (red) results obtained in the left ventricle ($r^2 = 0.78$).*

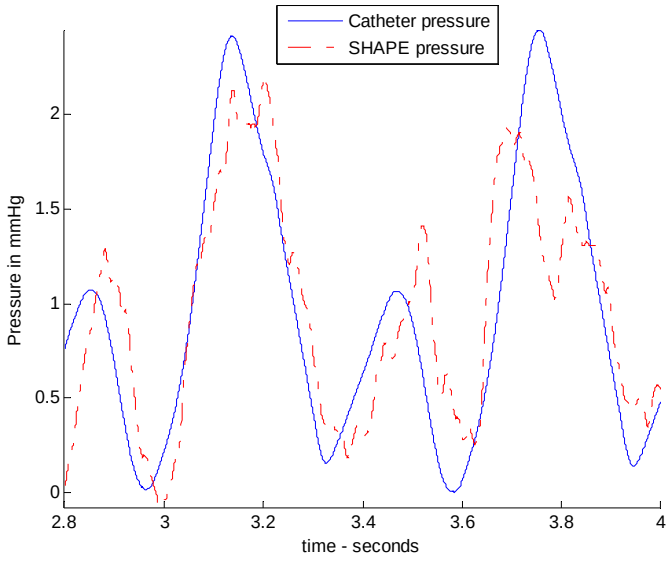

Figure 4. *Comparison of the catheter pressure (blue) and SHAPE pressure (red) results obtained in the right atrium (r^2 = 0.66).*

Initial experiments conducted to test the utility of two probes, L14-5/38 and L9-4/38 (Ultrasonic Medical Corp.), for *in vivo* human studies proved inconclusive as neither probe showed a clear subharmonic peak over the noise level. This was the case for both the PIHI and the non-PIHI (i.e., conventional) mode. Currently further testing to resolve this aspect is ongoing. These experiments represent the commencement of task 2a.

6 KEY RESEARCH ACCOMPLISHMENTS

- SHAPE experiments were conducted with Definity and Sonazoid *in vitro* in a pressurized watertank (range: 0-50 mmHg).
- SHAPE experiments were conducted *in vitro* with Definity using the Sonix RP scanner and two different probes; L14-5/38 and L9-4/38.
- Hydrostatic pressure is inversely related to the change in subharmonic amplitude (r^2 = 0.76–0.91, $p < 0.01$).
- A computer model to simulate the behavior of microbubbles as a function of pressure has been developed.
- Software for processing of *in vivo* SHAPE data has been developed.
- The possibility of *in vivo* human studies of SHAPE in patients with LABC using Definity is being explored.

7 REPORTABLE OUTCOMES

Publications

F. Forsberg, A. Katiyar, L. M. Leodore, K. Sarkar. Noninvasive subharmonic pressure estimation: in vitro experiments and modeling. *J Ultrasound Med*, vol 28 (Suppl.), pp. S120 – S121, 2009.

F. Forsberg, V. G. Halldorsdottir, J. Dave, M. McDonald, J. B. Liu, C. Leung, K. Dickie. In vivo noninvasive cardiac subharmonic pressure estimation. *Ultrasonic Imaging,* vol. 31, pp. 45-46, 2009.

V. G. Halldorsdottir, L. Leodore, B. Cavanaugh, F. Forsberg. Pressure estimation for monitoring neoadjuvant chemotherapy of breast cancer: In vitro measurements. *Ultrasonic Imaging,* vol. 31, pp. 46, 2009.

F. Forsberg. Applications of subharmonic contrast imaging. *Ultrasound Med Biol*, vol. 35, pp. S32, 2009.

F. Forsberg, J. Dave, V. G. Halldorsdottir, M. McDonald, J. B. Liu C. Leung, K. Dickie. Noninvasive subharmonic pressure estimation in vivo and in vitro. Accepted for publication in *Proc ICUS*, 2009.

V. G. Halldorsdottir, L. M. Leodore, B. Cavanaugh, F. Forsberg. Initial in vitro study of US pressure measurements for monitoring neoadjuvant chemotherapy of breast cancer. Accepted for publication in *Prog. RSNA*, 2009.

Presentations

April 2 - 5, 2009	The 54th Annual Convention of the American Institute of Ultrasound in Medicine, New York City, NY, USA. • Noninvasive subharmonic pressure estimation: in vitro experiments and modeling.
June 10 – 12, 2009	34th International Symposium on Ultrasonic Im aging and Tissue Characterization, Arlington, VA. • Pressure estimation for monitoring neoadjuvant chemotherapy of breast cancer: in vitro measurements. • In vivo noninvasive cardiac subharmonic pressure estimation.
August 30 - September 3, 2009	The 12th Congress of the World Federation for Ultrasound in Medicine and Biology, Sydney, Australia. • Applications of subharmonic contrast imaging.

8 CONCLUSIONS

Definity as well as Sonazoid showed an inverse linear relationship between the change in subharmonic amplitude and hydrostatic pressure ($r^2 = 0.76–0.91$, $p < 0.01$) over the pressure range associated with breast tumors (0 – 47 mmHg). These results indicate that the use of SHAPE for noninvasive evaluation of the IFP in breast lesions is feasible with both Definity and Sonazoid. However, difficulty in aligning the setup has delayed the project by approximately 6 months.

Our attempts to design a realistic simulation model accounting for the experimental results have been mixed and further work is ongoing. Software for analyzing RF data from the Sonix RP scanner to produce SHAPE pressure estimates has been successfully developed.

In summary, task 1 has been partially completed while tasks 2a and 2b are ongoing, but due to the delay caused by the *in vitro* experiments and the efforts invested in potentially acquiring human data (at no expense to this grant), the project is approximately 6 months behind schedule.

9 REFERENCES

1. Favret AM, et al., *Locally Advanced Breast Cancer: Is Surgery Necessary?* The Breast Journal, 2001. **7**(2): p. 131-137.
2. Kaufmann M, et al., *International Expert Panel on the Use of Primary (Preoperative) Systemic Treatment of Operable Breast Cancer: Review and Recommendations.* J Clin Oncol, 2003. **21**(13): p. 2600-2608.
3. Guarneri V, et al. *Primary systemic therapy for operable breast cancer: A review of clinical trials and perspectives.* Cancer Letters, 2007. **248**(2): p. 175-185.
4. Esteva, FJ, Hortobagyi GN, *Integration of Systemic Chemotherapy in the Management of Primary Breast Cancer.* Oncologist, 1998. **3**(5): p. 300-313.
5. Waljee JF, Newman LA, *Neoadjuvant Systemic Therapy and the Surgical Management of Breast Cancer.* Surgical Clinics of North America, 2007. **87**(2): p. 399-415.
6. McMasters KM, Hunt KK, *Neoadjuvant Chemotherapy, Locally Advanced Breast Cancer, and Quality of Life.* J Clin Oncol, 1999. **17**(2): p. 441-.
7. Mauri D., Pavlidis N, and Ioannidis JPA, *Neoadjuvant Versus Adjuvant Systemic Treatment in Breast Cancer: A Meta-Analysis.* J. Natl. Cancer Inst., 2005. **97**(3): p. 188-194.
8. Heldin CH, et al., *High interstitial fluid pressure - an obstacle in cancer therapy.* Nat Rev Cancer, 2004. **4**(10): p. 806-13.
9. Less JR, et al., *Interstitial hypertension in human breast and colorectal tumors.* Cancer Res, 1992. **52**(22): p. 6371-4.
10. Taghian AG, et al., *Paclitaxel Decreases the Interstitial Fluid Pressure and Improves Oxygenation in Breast Cancers in Patients Treated With Neoadjuvant Chemotherapy: Clinical Implications.* J Clin Oncol, 2005. **23**(9): p. 1951-1961.

11. Milosevic M, et al., *Interstitial fluid pressure predicts survival in patients with cervix cancer independent of clinical prognostic factors and tumor oxygen measurements.* Cancer Res, 2001. **61**(17): p. 6400-5.
12. Goldberg BB, Liu JB, Forsberg F, *Ultrasound contrast agents: A review.* Ultrasound in Medicine & Biology, 1994. **20**(4): p. 319-333.
13. Shi WT, Forsberg F, et al., *Pressure dependence of subharmonic signals from contrast microbubbles.* Ultrasound in Medicine & Biology, 1999. **25**(2): p. 275-283.
14. Ishihara K, et al., *New Approach to Noninvasive Manometry Based on Pressure Dependent Resonant Shift of Elastic Microcapsules in Ultrasonic Frequency Characteristics* Japanese Journal of Applied Physics, 1988. **27**(Suppl. 27-1): p. 125-127.
15. Forsberg F, Shi WT, Goldberg BB, *Subharmonic imaging of contrast agents.* Ultrasonics, 2000. **38**(1-8): p. 93-98.
16. Sarkar K, Shi WT, Chatterjee D, Forsberg F. *Characterization of ultrasound contrast microbubbles using in vitro experiments and viscous and viscoelastic interface models for encapsulation.* J Acoust Soc Am, **118**:539-550, 2005.
17. Halldorsdottir VG, Leodore LM, Cavanaugh B, Forsberg F. *Pressure estimation for monitoring neoadjuvant chemotherapy of breast cancer: In vitro measurements* (abstract). Ultrasonic Imaging, **31**: 46, 2009.
18. Halldorsdottir VG, Leodore LM, Cavanaugh B, Forsberg F. *Initial in vitro study of US pressure measurements for monitoring neoadjuvant chemotherapy of breast cancer* (abstract). Accepted for publication in Prog. RSNA, 2009.
19. Forsberg F, Katiyar A, Leodore LM, Sarkar K. *Noninvasive subharmonic pressure estimation: in vitro experiments and modeling* (abstract). J Ultrasound Med, **28:** S120 – S121, 2009.
20. Forsberg, Halldorsdottir VG, Dave J, McDonald M, Liu JB, Leung C, Dickie K. *In vivo noninvasive cardi iac subharmonic pressure estimation* (abstract). Ultrasonic Imaging, **31**:45-46, 2009.

Appendix I

The Statement of Work from the original proposal:

Objective 1
Task 1: Computer modeling and *in vitro* experiments (months 1 - 6)

a. Construct an *in vitro* experimental pulse-echo system for investigating the effect of hydrostatic pressure variations on contrast microbubbles and measuring the resulting changes in backscattering (Month 1).
b. Design and modify numerical codes for a theoretical model describing the dynamics of contrast microbubbles under different pressure conditions (Months 1 - 3).
c. Calculate the behavior of individual contrast microbubble and the collective behavior of contrast microbubble populations (Months 3 - 6).
d. Measure changes in backscattered fundamental, second and subharmonic signals for different contrast agents as a function of pressure (Months 2 - 6).
e. Predict optimal contrast agents for SHAPE according to the numerical simulations (Month 6).
f. Select optimal contrast agent(s) for SHI and SHAPE. The selection will mainly be based on experimental measurements (Month 6).

Objectives 2 - 3
Task 2: Design and implementation of SHAPE on a commercial US scanner (months 7 - 12)

a. Optimize SHI and SHAPE, based on *in vitro* measurements and simulations using the actual parameters of the designated transducers (Months 7 – 8).
b. Modify a state-of-the-art US imaging system (the Sonix RP) to incorporate the SHI contrast imaging modality and to perform SHAPE (Months 8 - 10).
c. Evaluate the new imaging modality and SHAPE in an *in vitro* phantom using the modified US scanner (Months 11 - 12).
d. Prepare regulatory review and obtain approval for animal studies (Months 9 - 12).

Objectives 3 - 5
Task 3: Animal experiments, data collection and analysis (months 13- 36)

a. Create and grow breast tumors by implanting one of three human breast cancer cell lines (SKBR3, BT474 or MCF-7) into the mammary fat pad of athymic, nude rats (Months 13 - 34).
b. Calibrate *in vivo* SHAPE results based on IFP measurements obtained with the intra-compartmental pressure monitor in 21 nude rats. Three groups (one per cell line) of 7 rats with breast tumors implanted will be studied (months 14 - 16).

c. Produce and evaluate the ability of SHI to depict normal vascularity as well as breast tumor angiogenesis in human xenografts implanted in nude rats compared to CD31 stained specimens (Months 17 - 34).
d. Validate the clinical potential of SHAPE as a therapy monitoring tool by studying 180 human xenograft breast tumors in nude rats (42 normal rats and 138 after administration of a chemotherapy agent paclitaxel) and comparing results to intra-compartmental pressure measurements (months 17 - 34).
e. Perform statistical analyses and write final report (months 34 - 36).